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(54) Title: GENE SILENCING

(57) Abstract: Disclosed is a method for silencing the expression of an endogenous gene in a cell, the method involving overexpressing in the cell a nucleic acid molecule of the endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene, wherein the overexpression of the nucleic acid molecule of the endogenous gene and the antisense molecule in the cell silences the expression of the endogenous gene.

## GENE SILENCING

5

### Background of the Invention

This invention relates generally to the silencing of gene expression, as well as to expression vectors useful for silencing the expression of a target gene.

Cells manufacture proteins by transcribing genes encoded by DNA. In particular, one of the DNA strands of the gene is transcribed by an enzyme, RNA polymerase, to produce mRNA. The mRNA molecule has a base sequence that is complementary to that of the transcribed DNA strand. The mRNA is then processed by the removal of introns. The base sequence of the mRNA is next translated into the amino acid sequence of a protein molecule by means of the genetic code. This translation process requires many enzymes and a set of transfer RNA molecules, which align the amino acids according to the codon sequence. The translation of mRNA into protein occurs on ribosomes. The translated protein is often referred to as a gene product.

Developmental and physiological processes of cells and organisms require that the gene products necessary to carry out these processes are available in the appropriate amounts and at the appropriate times. For example, certain gene products must be present in all cells for many fundamental physiological processes to occur. Other gene products have tissue- or cell-specificity and are only necessary in certain cells or tissues. Some gene products are continuously present either in all cells or in certain cells or tissues. Still other gene products are required at different times during development.

Gene regulation, in both animals and plants, alters the quantity or quality or both of a gene product. These alterations can be used to ascertain the molecular activities of the normal gene product counterpart. Additionally, gene regulation can be used to manipulate cells and organisms at the genetic level. Gene regulation provides scientists with an expanded ability to study and treat disease processes. In particular, gene regulation techniques have proven especially useful in the elucidation

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and diagnosis of many diseases and abnormalities. Indeed, it is now possible to use gene regulation for therapeutic intervention and treatment at the genetic level. In addition, gene regulation can be used to identify and characterize genes involved in fundamental cellular and developmental processes of animals and plants. The

5 identification and characterization of these genes has previously been hampered by the fact that mutations in such genes are often lethal or are recessive in diploid organisms. Accordingly, the selective inactivation or regulation of genes has many potential uses.

Certain genes are regulated at the level of transcription. Transcriptional  
10 regulation is carried out either negatively (repressors) or positively (activators) by a protein factor. Specific protein factors regulate translation of specific mRNAs. It has also become evident that RNAs are involved in regulating the expression of specific genes.

Different approaches have attempted to regulate gene expression by  
15 selectively inactivating genes. For example, one approach to gene regulation is gene disruption. Gene disruption is accomplished using recombinant DNA techniques. It is generally a process of sequential elimination of the alleles for a particular gene. The alleles are eliminated by introducing a mutation into the gene, usually by homologous recombination, at the single cell stage of the organism, which renders the  
20 gene nonfunctional. Gene disruption is technically difficult and labor intensive. In addition, additional genes are sometimes unintentionally disrupted.

Another gene regulation approach involves the use of antisense RNA. Antisense RNA inhibition of specific mRNAs involves the use of DNA constructs that direct the transcription of an antisense RNA strand. For example, antisense DNA  
25 constructs have been prepared by flipping a gene fragment of interest and inserting this sequence between a promoter and a polyadenylation site in the inverse orientation. The RNA transcript obtained from this DNA molecule has a sequence complementary to a target mRNA. The antisense RNA anneals to the mRNA and disrupts normal processing or translation or both. Antisense constructs can be

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introduced into eukaryotic cells by standard transfection, transduction, or microinjection methods and function in both transient and stable transformation assays. Antisense transcripts complementary to the target gene mRNA specifically suppress gene activity.

5       Despite the availability of these and other methods for regulating gene expression, there exists a need for gene regulation methods that are capable of silencing or suppressing a specific gene product or multiple gene products.

#### Summary of the Invention

In one aspect, the invention features a method for silencing the expression 10 of a targeted gene (e.g., an endogenous gene) in a cell. The method generally involves overexpressing in the cell (e.g., an animal cell such as a mammalian cell, a cancer cell, or a plant cell) an isolated nucleic acid molecule of an endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene, wherein the overexpression of the 15 nucleic acid molecule of the endogenous gene and the antisense molecule in the cell silences the expression of the endogenous gene. Exemplary genes targeted for silencing include, without limitation, transcription regulatory factors, virally encoded proteins (e.g., human papilloma virus E6 and human immunodeficiency virus tat), structural proteins, metabolic or enzymatic proteins, cytokines, oncogenes, growth 20 factors (e.g., interleukins), gamma interferon, tumor necrosis factor, and granulocyte-macrophage-colony stimulating factor.

In preferred embodiments, the isolated nucleic acid molecule of an endogenous gene generates an untranslatable RNA molecule or a translatable RNA molecule that encodes a non-functional product. In other preferred embodiments, the 25 isolated nucleic acid molecule includes the coding region of the endogenous gene or a portion thereof. While in still other embodiments, the antisense molecule is complementary to a portion of the gene targeted for silencing.

Preferably, the isolated nucleic acid molecule of the endogenous gene used

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in the invention includes an exon (e.g., an exon that is between 20-2,000 base pairs). In preferred embodiments, overexpression of the exon and the antisense molecule silences the expression of a gene family. In other preferred embodiments, overexpression of the exon and the antisense molecule silences the expression of a 5 specific member of the gene family.

In yet other preferred embodiments, the expression of the isolated nucleic acid molecule, antisense molecule, or exon is controlled by an inducible expression control region, a tissue- or cell-specific expression control region, or by a (fully or 10 partially) constitutive expression control region.

In preferred embodiments, the endogenous gene selected for gene silencing encodes a protein, for example, a metabolic enzyme, a structural protein, or a gene product that is associated with a disorder (e.g., an autoimmune disease, cancer, tissue inflammation, or a dysfunction in a metabolic pathway). In still other preferred 15 embodiments, the endogenous gene encodes a gene product that when silenced confers on a plant an agronomically important trait (e.g., fruit ripening, senescence, male sterility, wounding response, disease resistance, or dessication tolerance).

In another aspect, the invention features a method for silencing the expression of an endogenous gene in a cell. The method generally involves 20 expressing in the cell a vector including (i) an expression control region functional in the cell; and (ii) an operably linked DNA molecule including a proximal region and a distal region, the proximal region having substantial sequence identity to an endogenous gene of the cell targeted for silencing, wherein transcription of the DNA generates an RNA molecule having a genetically-engineered dsRNA stem-loop 25 structure based on complementarity between nucleotides found in the distal region of the RNA molecule. In preferred embodiments, the expression vector is overexpressed in the cell.

In still another aspect, the invention features a gene silencing expression vector including (i) an expression control region functional in the cell; and (ii) an

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- operably linked DNA molecule including a proximal region and a distal region, the proximal region having substantial sequence identity to an endogenous gene of the cell targeted for silencing, wherein transcription of the DNA generates an RNA molecule having a genetically-engineered dsRNA stem-loop structure based on complementarity between nucleotides found in the distal region of the RNA molecule.
- 5

In another aspect, the invention features a method for silencing the expression of an endogenous gene in a cell. The method generally involves expressing in the cell a vector including: (i) an expression control region functional in the cell; and (ii) an operably linked DNA molecule having substantial sequence identity to an endogenous gene of the cell; wherein transcription of the DNA generates an RNA molecule that forms dsRNA structure based on complementarity between nucleotides found in the 5' and 3' regions of the RNA molecule.

10

In yet another aspect, the invention features a gene silencing expression vector that includes: (i) an expression control region functional in the cell; and (ii) an operably linked DNA molecule having substantial sequence identity to an endogenous gene of the cell; wherein transcription of the DNA generates an RNA molecule that forms a dsRNA structure based on complementarity between nucleotides found in the 5' and 3' regions of the RNA molecule.

15

In still another aspect, the invention features transgenic plants and non-human transgenic animals that overexpress in a cell (e.g., a non-human animal cell such as a mammalian cell, a cancer cell, or a plant cell) an isolated nucleic acid molecule of an endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene. The invention also features transgenic plants and non-human transgenic animals including the expression vector of the invention.

20

As used herein, by "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By "substantially identical" is meant a nucleic acid molecule exhibiting at

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- least 40%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% contiguous sequence identity to a reference sequence. The length of comparison sequences will generally be at least 15-20 nucleotides, preferably at least 50 nucleotides, more preferably at least 75 nucleotides, and most preferably 100
- 5 nucleotides or more. Sequence identity is measured, for example, using standard sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, Blast, BlastN, BlastP, BlastX, FastA, or PILEUP/Prettybox programs) set to standard parameters.
- 10 By "nucleic acid" or "nucleic acid molecule" is meant a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger expression vector. For example, nucleic acids can be assembled from cDNA fragments or from oligonucleotides to generate a synthetic gene which is capable of being expressed in a recombinant transcriptional unit,
- 15 generating transcripts that accumulate stably. Polynucleotide or nucleic acid molecules of the invention therefore include DNA, RNA, cDNA, or synthetic nucleic acid sequences.

By "isolated nucleic acid molecule" is meant a nucleic acid molecule (e.g., DNA) that is free of the genes which, in the naturally-occurring genome of the

20 organism from which the nucleic acid molecule is derived, flank the gene. The term therefore includes, for example, a gene or fragment thereof that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease

25 digestion or a synthetic nucleic acid molecule) independent of other sequences. It also includes a recombinant nucleic acid molecule which is part of a hybrid gene encoding additional polypeptide sequence.

By "antisense molecule" is meant an RNA molecule that includes a nucleotide sequence that is complementary to a messenger RNA. In general, such an

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antisense molecule will usually be at least 15 nucleotides, preferably, about 15-200 nucleotides, and, more preferably, 200-2,000 nucleotides in length. The antisense sequence may be complementary to all or a portion of the mRNA nucleotide sequence, and, as appreciated by those skilled in the art, the particular site or sites to which the antisense sequence binds as well as the length of the antisense sequence will vary, depending upon the degree of inhibition desired and the uniqueness of the antisense sequence. Antisense molecules may be constructed and expressed as described herein or as described, for example, in van der Krol et al., Gene 72, 45 (1988); Rodermel et al., Cell 55, 673 (1988); Mol et al., FEBS Lett. 268: 427 (1990); Weigel and Nilsson, Nature 377, 495 (1995); Cheung et al., Cell 82, 383 (1995); and U.S. Pat. No. 5,107,065.

By "obtained from" is meant isolated from or having the sequence of a naturally-occurring sequence (e.g., a cDNA, genomic DNA, synthetic DNA, or combination thereof).

By "expression control region" is meant any minimal sequence sufficient to direct transcription in a host cell (e.g., a plant or animal cell). Included in the invention are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress-, or hormone-inducible elements or chemical inducers) or elements that are capable of cycling gene transcription; such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

By "exon" is meant any length of nucleic acid sequence, excluding intron sequences and excluding the non-coding regulatory sequences driving transcription. The exon sequence may be obtained in whole or in part from any source known in the art, including a plant, a fungus, an animal, a bacterial genome or episome, eukaryotic, nuclear or plasmid DNA, cDNA, viral DNA, or chemically synthesized DNA. If desired, an exon sequence of a target gene may contain one or more modifications in either the coding or the untranslated regions which affect the biological activity or the

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chemical structure of the expression product, the rate of expression, or the manner of expression control. Such modifications include, but are not limited to, mutations, insertions, deletions, and substitutions of one or more nucleotides.

By "structural gene" is meant an uninterrupted sequence of a nucleic acid  
5 molecule, including one or more introns, bound by the appropriate splice junctions.

By "operably linked" is meant that a gene and a regulatory sequence(s) (e.g., an expression control region) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

10 By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and  
15 microspores.

By "crucifer" is meant any plant that is classified within the Cruciferae family. The Cruciferae include many agricultural crops, including, without limitation, rape (for example, *Brassica campestris* and *Brassica napus*), broccoli, cabbage, brussel sprouts, radish, kale, Chinese kale, kohlrabi, cauliflower, turnip, rutabaga,  
20 mustard, horseradish, and *Arabidopsis*.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an  
25 endogenous gene of the organism.

By "transgenic" is meant any cell which includes a nucleic acid molecule (e.g., a recombinant DNA molecule) which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants or non-human

animals, and the DNA (transgene) is inserted by artifice into a nuclear or plastidic genome.

By "introducing into an animal or plant a nucleic acid" includes any number of ways of introducing a gene sequence into a eukaryotic cell that include, 5 without limitation, retroviruses, transfection, liposomes, *Agrobacterium*-mediated transformation, and biolistics, such that the introduced gene is maintained as an episome or incorporated into the genome of the cell, and is maintained and replicates as the cell divides and replicates.

By "gene therapy" is meant any therapy by which a biological deficit is 10 repaired by the introduction of a nucleic acid sequence that encodes a gene product capable of improving or delaying the disease.

By "target gene" is meant a gene that is pre-selected for gene silencing.

By "silencing" is meant that the expression of a gene is inhibited, suppressed, or decreased. Preferably, a gene is considered silenced when its 15 expression is inhibited, suppressed, or decreased, for example, by at least 50% of the normal expression level, preferably by at least 75%, and more preferably by at least 90%.

By "genetically-engineered" is meant to splice, modify, or alter a nucleic acid molecule using standard recombinant molecular techniques or methodologies. A 20 DNA molecule, for example, may be modified or altered to include site specific mutations into one or more coding and/or noncoding regions which result, upon transcription, in the production of an RNA molecule having a stem-loop structure. Such techniques may also be used to engineer a mutation, including, but not limited to an insertion, deletion, or substitution of one or more nucleotides of a nucleic acid 25 molecule (e.g., a DNA molecule).

By "agronomically important trait" is meant a trait or characteristic found in a plant that contributes to its economic value. Such traits include, without limitation, stress tolerance or resistance or both, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative

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stress; increased yields; food content and makeup; physical appearance; male sterility; starch properties; quantity and quality of secondary compounds; sugar quantity and quality; oil quantity and quality; and protein quantity and quality. Accordingly, one may desire to silence one or more genes involved in a regulatory or biosynthetic

- 5 pathway as a means of conferring any such desirable agronomically important trait or traits on a plant.

The invention is useful for modifying the activity of an enzyme; to provide for the specific or preferential expression of an allele or one or members of a multigene family; or for the expression of a particular isozyme. In addition, the 10 invention is useful for modulating metabolic pathways or plant development.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Detailed Description

The drawing will first be described.

15 **Drawing**

Figure 1 shows exemplary mechanisms for utilizing dsRNA-based gene silencing for the synthesis of cRNA (complementary RNA). The cRNA is typically not capped and does not have include a polyA<sup>+</sup> tail, which enhances the degradation of the mRNA encoded by a preselected target gene, and for its transport through 20 phloem and plasmodesmata of plants.

Figure 2 is a photograph showing the effects of overexpressing sense and antisense *AtHXP1* genes in transgenic *Arabidopsis* seedlings germinated on 7% glucose/MS. The transgenic seedling expressing both sense and antisense constructs is shown on the left; the seedling expressing sense *AtHXP1* is shown on the right.

25 **Gene Silencing Expression Vectors**

The present invention includes novel nucleic acid expression vectors which provide a general means for silencing the expression of pre-selected target

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genes. The expression vectors of the invention generally include an expression control region and an isolated nucleic acid molecule (e.g., a DNA molecule) having substantial sequence identity to a gene whose expression is selected to be silenced (e.g., an endogenous gene). Upon transcription, the DNA molecule generates an RNA 5 molecule. A portion of this generated RNA molecule then folds into a double-stranded (ds) RNA stem-loop or a region of dsRNA. Also included in the invention is a method of silencing gene expression utilizing the expression vector of the invention.

Nucleic acid molecules utilized in the invention can be obtained by several methods. Sequences for specific genes or stem-loop structures, for example, can be 10 obtained from published sequences and can also be found in the GenBank computer database (Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>), or may be determined after isolation using standard techniques. Nucleic acids can then be chemically synthesized, if desired, by standard methods.

#### Double-Stranded RNA Stem-Loops

15 As is discussed above, the expression vector of the invention which is useful for silencing the expression of an endogenous gene or virtually any other targeted gene includes a DNA molecule that, upon transcription, generates an RNA molecule having a stem-loop structure. Preferably, the stem-loop structure, within the generated RNA molecule, is positioned distally relative to the region of the RNA 20 molecule encoding the sequence of the gene selected for silencing. While not wanting to be bound by a particular theory, it is believed that the stem-loop structure provides a binding site or template for a dsRNA-dependent RNA polymerase. dsRNA-dependent RNA polymerase in turn synthesizes a cRNA based on the RNA template of the targeted gene. Exemplary strategies for designing expression constructs that 25 express RNA molecules useful for the synthesis of cRNA are shown in Fig. 1. The synthesis of the cRNA is thought to promote RNA degradation and concomitant silencing of the target gene.

The stem-loop structure refers to a nucleic acid structure that folds preferably into a hairpin form. Stem-loop structures used in the expression vectors of

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the invention are typically unmodified, naturally-occurring structures, and are readily engineered for incorporation into the expression vector using standard cloning techniques. Alternatively, standard methods may be employed to synthesize stem-loop structures that mimic the naturally-occurring structures. Such genetically  
5 engineered stem-loop structures, like naturally-occurring stem-loop structures, can then be integrated into the expression vector of the invention. It will be understood that the folding pattern of the stem-loop structure is not compromised by alterations in the nucleic acid sequence of the naturally-occurring molecule. For example, it is understood that alterations which include, but are not limited to, mutations, insertions,  
10 deletions, and substitutions of one or more nucleotides can be made within the sequence of the stem-loop, as long as the hairpin formation of the stem-loop is maintained. Hairpin structures useful in the invention are preferably 100-200 base pairs or more in length.

In addition, a DNA molecule may be genetically engineered to express an  
15 RNA molecule that forms a dsRNA structure based on complementarity found between nucleotides in its 5' and 3' regions (Fig. 1). Preferably, the dsRNA structure is approximately 20-50 bp, or is of a size sufficient to provide a template for a dsRNA-dependent RNA polymerase. The remaining non-complementary region of the RNA molecule has substantial identity to a sequence encoding a preselected target  
20 gene.

dsRNA-dependent RNA polymerase

Constructs expressing a dsRNA-dependent RNA polymerase may also be introduced in a cell according to standard methods known in the art. Exemplary polymerases are described in Schiebel et al. (Plant Cell 10: 2087, 1998) and Cogoni et  
25 al. (Nature 399: 166, 1999). Expression of a dsRNA-dependent RNA polymerase facilitates RNA degradation and, consequently, gene silencing. For example, if a cell does not express an endogenous dsRNA-dependent RNA polymerase or expresses an insufficient level of the enzyme, a heterologous dsRNA-dependent RNA polymerase

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from any number of organisms (e.g., a plant) may be introduced to promote the generation of sequence-specific cRNAs.

Target Sequences

- The expression vector of the invention also includes an isolated nucleic acid molecule (e.g., a DNA molecule) that encodes an RNA molecule corresponding to a pre-selected target gene (e.g., an endogenous gene of a cell) whose expression is to be silenced. Thus, the nucleic acid molecule once expressed in the cell silences the expression of virtually any target gene. For example, if hexokinase gene expression is to be silenced in a cell, then the expression vector of the invention incorporates a DNA molecule that is substantially identical to an endogenous hexokinase gene of the cell.

- The DNA molecule utilized in the expression vector of the invention is generally obtained directly from the target gene that is to be silenced and differs, as is discussed above, primarily by the inclusion of a region which, upon transcription, folds into a dsRNA stem-loop structure. Furthermore, the DNA molecule, if desired, may be chemically synthesized. The DNA molecule encoding the RNA molecule has generally over 90% identity to the target gene to be silenced. However, DNA molecules having less than 90% identity to the target gene sequence may also be utilized; for example, a DNA molecule encoding an RNA molecule will also be effective when it shares at least 80% sequence identity with the target gene to be silenced.

- While a DNA molecule may encode an RNA molecule corresponding to the full length sequence of the target gene, substantially less than the full length of the target gene can be used. For example, a DNA molecule corresponding to a portion of the target gene may also be utilized. Accordingly, relatively short regions of the target gene may be used to engineer a DNA molecule depending on the size of the target gene to be silenced.

In particular examples, in some applications, the DNA molecule of the expression vector may be 15-200 nucleotides in length, although longer sequences,

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such as 100-250 nucleotides may also be utilized. In such applications where such shorter sequences (e.g., less than 200 nucleotides) are utilized, the introduced DNA molecule will share a high degree of identity with the target gene sequence, being preferably at least 80% or even 90% identical. In other applications, a sequence of  
5 greater than 500 nucleotides may be used, again depending on the size of the target gene to be silenced.

Thus, the DNA molecule employed in the expression vector of the invention generally includes one contiguous nucleic acid sequence of at least 15-20 nucleotides in length that has at least 80% identity to a sequence found in the target  
10 gene to be silenced. In other applications, the DNA molecule includes at least one contiguous nucleic acid sequence of at least 100 nucleotides in length that has at least 80% identity to the target gene. And, in still other applications, the DNA molecule includes at least one contiguous nucleic acid sequence of at least 250 nucleotides in length that has at least 85% identity to the target gene.

15 Because a full length sequence is unnecessary, the invention is not limited to the silencing of individual target genes. It is also possible to silence the expression of multiple genes using a single introduced DNA molecule that contains sequences corresponding to several genes (e.g., members of a multigene family) that are expressed in the target cell. Alternatively, it is possible to silence the expression of  
20 specific members of a multigene family using a single introduced DNA molecule that contains a gene-specific sequence (e.g., sequences typically found in the 3' untranslated regions of a mRNA transcript) corresponding to the specific gene family member to be silenced in the target cell.

Once a target gene to be silenced is identified, a DNA molecule having  
25 substantial identity to the target gene sequence is manipulated to render the RNA molecule encoded by the DNA molecule to include a stem-loop structure, using standard cloning methodologies. Furthermore, the proximal region of the DNA molecule itself may be tailored and manipulated using standard *in vitro* mutagenesis (e.g., using those methods described below) to modify the molecule so that upon

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transcription stable dsRNA stem-loop structure formation is facilitated.

Additionally, the RNA molecule encoded by the DNA molecule may be rendered untranslatable by employing standard methods. For example, the translation initiation codon (e.g., an ATG) can be removed from the DNA sequence.

- 5 Alternatively, additional translational initiation codons may be introduced into the DNA molecule. Furthermore, the reading frame of the DNA sequence can be displaced by the addition of one or more bases. Stop codons may also be introduced after initiation codons. Many standard DNA mutagenesis techniques are available to introduce such modifications into the DNA molecule including, for example, M13 primer mutagenesis. Details of such techniques are provided in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY (1998).

Expression Control Regions

- 15 A wide range of expression control regions may be utilized in the expression vector of the invention, including viral, plant, or mammalian promoters. Additionally, cell- or tissue-specific promoters can, if desired, be used to trigger expression of a nucleic acid molecule in specific cell populations.

- 20 Mammalian and viral promoters suitable for use in the present invention are available in the art. The choice of a particular promoter as an expression control region of the invention, is dictated by the spatial and temporal pattern of expression that is desired for the expression of the target gene encoded by the DNA molecule of the expression vector, and ultimately for the location where the target gene is to be silenced. Expression control regions of the invention therefore include eukaryotically derived promoters which predominantly direct expression in, for example, the reproductive system (e.g., breast, ovary, testes); the musculoskeletal system (e.g., muscle or joint tissue); the cardiovascular system (e.g., capillaries or heart); the respiratory system (e.g., lung or nasal passages); the urological system (e.g., kidney or bladder); the gastrointestinal system (e.g., pancreas, liver, or intestines); the immune

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system (e.g., thymus, spleen, or circulating immunological cells); the endocrine system (e.g., pituitary, gonads, and thyroid); the nervous system (e.g., neurons); or the hematopoietic system (e.g., bone marrow and peripheral blood). Further, promoters of the invention include, but are not limited to, the elastase promoter (expression in 5 pancreatic acinar cells); the alpha-A-crystallin promoter (expression in the eye lens tissue); the insulin promoter (expression in the pancreatic beta cells), and the albumin promoter regions.

Additionally, non-eukaryotically-derived promoters such as virally-derived and prokaryotically-derived promoters are included in the present invention. Such 10 promoters include, but are not limited, to the mouse mammary tumor virus promotor (MMTV), SV40 early region promoter, Rous sarcoma virus (RSV) promoter, or cytomegalovirus (CMV) promoter, which direct expression of viral or host genes in specific tissues and in many cell types.

In addition to the above-described expression control regions, a number of 15 gene promoters which are active in plant cells have been described in the literature. An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for 20 both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odell et al., Nature 313:810, 1985). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, 1990; Terada and Shimamoto, Mol. Gen. Genet. 220:389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) 25 by duplication of the CaMV 35S promoter (see e.g., Kay et al., Science 236:1299, 1987; Ow et al., Proc. Natl. Acad. Sci., U.S.A. 84:4870, 1987; and Fang et al., Plant Cell 1:141, 1989). In addition, a minimal 35S promoter may also be used as is described herein.

Other useful plant promoters include, without limitation, the nopaline

synthase promoter (An et al., *Plant Physiol.* 88:547, 1988) and the octopine synthase promoter (Fromm et al., *Plant Cell* 1:977, 1989). Exemplary monocot promoters include, without limitation, commelina yellow mottle promoter, sugar cane badna virus promoter, rice tungro baciliform virus promoter, maize streak virus element, and 5 wheat dwarf virus promoter.

For certain applications in plants, it may be desirable to express the DNA molecule of the expression construct in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For these purposes, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its 10 regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for heat-regulated gene expression (see, e.g., Callis et al., *Plant Physiol.* 88:965, 1988; Takahashi and Komeda, *Mol. Gen. Genet.* 219:365, 1989; and Takahashi et al., *Plant J.* 2:751, 1992), light-regulated gene expression (e.g., the 15 *Arabidopsis Cab2* photosynthetic, leaf specific promoter described by Mitra et al., *Plant Mol. Biol.* 12: 169-179, 1989; the pea *rbcS-3A* described by Kuhlemeier et al., *Plant Cell* 1:471, 1989; the maize *rbcS* promoter described by Schäffner and Sheen, *Plant Cell* 3:997, 1991; or the chlorophyll a/b-binding protein gene found in pea described by Simpson et al., *EMBO J.* 4:2723, 1985), hormone-regulated gene 20 expression (for example, the abscisic acid (ABA) responsive sequences from the *E<sub>m</sub>* gene of wheat described by Marcotte et al., *Plant Cell* 1:969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and *Arabidopsis* by Straub et al., *Plant Cell* 6:617, 1994, Shen et al., *Plant Cell* 7:295, 1995; and wound-induced gene expression (for example, of *wunI* described by 25 Siebertz et al., *Plant Cell* 1:961, 1989), organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., *EMBO J.* 6:1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al., *EMBO J.* 7:1249, 1988; or the French bean  $\beta$ -phaseolin gene described by Bustos et al., *Plant Cell* 1:839, 1989; the vegetative storage protein promoter (soybean *vspB*) described

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by Sadka et al (Plant Cell 6: 737-749, 1994)), cycling promoters (e.g., the *Arabidopsis* cdc2a promoter described by Hemerly et al., Proc Natl Acad Sci USA 89: 3295-3299, 1992), senescence-specific promoters (e.g., the *Arabidopsis* SAG12 promoter described by Gan et al, Science: 270, 1986-1988, 1995), seed-specific promoters (for example, endosperm-specific or embryo-specific promoters), meristem-specific promoters (for example, the *Tch4* promoter described by Xu et al., Plant Cell 7:1555-67, 1995), or pathogen-inducible promoters (for example, PR-1 or  $\beta$ -1,3 glucanase promoters).

Transformation

Upon construction of the expression vector of the invention, several standard methods are available for introduction of the vector into a host cell (e.g., a plant or an animal). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. As newer methods are available to transform host cells, they may be directly applied.

15 Gene Silencing By Overexpression of Sense and Antisense RNAs

Gene silencing is also accomplished by the simultaneous overexpression of sense and antisense RNA transcripts having substantial identity to a gene targeted for regulation. The invention therefore includes a method of silencing a gene by introducing into a cell a sense DNA construct of the targeted gene and its antisense counterpart. Using this simple approach, virtually any gene (e.g., an endogenous gene) can be regulated by the methods of the invention.

Expression vectors designed to overexpress sense and antisense transcripts of a gene targeted for regulation are constructed according to standard methods (e.g., those described herein). For example, a eukaryotic expression vector system (e.g., a cell- or tissue-specific vector) may be used to express a DNA molecule (in the sense or antisense orientation on either the same or individual vectors). Appropriate gene sequences are introduced into a plasmid or other vector, which construct is then used to transform living cells according to standard methods. Constructs containing the

entire open reading frame, inserted as a translatable or untranslatable transcript, may be used. Alternatively, portions of a DNA molecule, may be inserted. One of skill in the art will readily be able to determine which sequences to use as the appropriate sense and antisense nucleic acid construct.

5       The gene silencing effect on a targeted gene using the methods of the invention is typically monitored using standard methods. For example, the effectiveness of the simultaneous overexpression of sense and antisense genes in silencing gene expression can be determined phenotypically or by standard Northern blot analysis or immunohistochemically. Other standard nucleic acid detection  
10 techniques or alternatively immunodiagnostic techniques known to those of skill in the art (e.g., Western or Northwestern blot analysis) may be employed. Typically, a gene is considered silenced when its expression is suppressed or inhibited for example, by at least 50% of the normal expression, preferably by at least 75%, and more preferably by at least 90%.

15       The following example is provided for the purpose of illustrating the invention, and should not be construed as limiting.

To demonstrate the application of the invention, a transgenic plant was generated which overexpressed both a sense transcript and an antisense transcript (~ 1,500 base pairs (bp)) complementary to the sense transcript. Using the hexokinase 20 (HXK) *AtHXK1* gene of the crucifer *Arabidopsis*, sense and antisense expression constructs were generated as described in Sheen et al., W097/35965. Gene constructs overexpressing either the sense or antisense RNAs of the coding sequences from the HXK1 gene were introduced into separate *Arabidopsis* lines. These lines were then crossed to produce plants overexpressing both sense and antisense RNAs. Plants resulting from the crosses displayed a stronger glucose insensitive phenotype than control plants. The HXK mutant was easily identified based on its ability to develop roots and expanded green cotyledons (Fig. 2). Additionally, these new HXK mutants were observed to display a stronger glucose insensitive phenotype than the null 25 *AtHXK1* mutant or the antisense *AtHXK1* transgenic plant. The glucose insensitive

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phenotype was taken as an indication that the expression of the HXK gene was silenced or knocked out by formation of double-stranded (ds) HXK RNA and resultant silencing of the endogenous *Arabidopsis* HXK gene. Finally, although plants overexpressing sense *AtHXK1* were found to have 20-50 times more AtHXK1 protein, no AtHXK1 protein was observed in plants overexpressing both the sense and antisense *AtHXK1* constructs. Similar results could be obtained by introducing sense and antisense DNA constructs in one expression vector, and introducing such an expression vector into a plant using standard methods.

In another example of simultaneously expressing sense and antisense constructs in the same plant, transgenic *Arabidopsis* plants expressing a full-length CDPK1 (calcium dependent protein kinase 1) cDNA were first generated as described in Sheen, WO 98/26045, using kanamycin as a selectable marker. Plants overexpressing the CDPK1 cDNA were observed as drought resistant. A homozygous sense CDPK1 T3 line was then generated, and two different antisense CDPK1 constructs (~ 250 bp and 700 bp), that shared identity between the CDPK1 and CDPK1a genes, were separately introduced into this line using standard *Agrobacterium* mediated plant cell transformation. Transgenic plants expressing both the sense and antisense constructs were then identified using bar and kanamycin selection. In plants expressing both sense and antisense constructs, the drought resistance phenotype was eliminated, and these plants were further found to be more sensitive to drought conditions than wild type plants. In addition, plants expressing both sense and antisense constructs were observed to have late flowering and aerial leaf phenotypes.

25 Uses

The invention described herein is useful for a variety of agricultural, horticultural, and medicinal purposes. For example, the expression vector(s) and methods described above are useful for silencing the expression of virtually any targeted gene or a particular metabolic pathway.

- In the agricultural and horticultural fields, for example, the invention is particularly useful for silencing the expression of a variety of genes including, without limitation, genes associated with, for example, agronomically important traits, such as the synthesis or metabolism of peptides, proteins, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, fragrances, toxins, carotenoid pigments, hormones, cell wall polymers, gene regulatory molecules, flavonoids, storage proteins, phenolic acids, coumarins, alkaloids, quinones, lignins, glucosinolates, tannins, aliphatic amines, celluloses, polysaccharides, glycoproteins, and glycolipids.
- For instance, an alteration in the production of fatty acids or lipids can be engineered (and fatty acid composition of, e.g., an oil-producing plant thus altered) by blocking synthesis of a specific chain elongation or desaturation enzyme. Also, the synthesis of starch and sugars can be reduced (and sugar content of, e.g., an edible plant thus altered) by blocking enzymes required for starch and carbohydrate synthesis. In addition, ethylene production can be blocked to delay senescence. Similarly, fragrant molecules can be released from cells (thus altering the scent characteristics of, e.g., ornamental flowers) by blocking the enzymes responsible for glycosylation of such molecules. In addition, male-sterile plants may be engineered by silencing genes responsible for the formation of the male gametes.
- In medicine, the invention provides a method of gene therapy for the treatment of cell proliferative or immunologic disorders and diseases such as those that are mediated by various overexpressed proteins. Exemplary cell-proliferative disorders include malignant as well as non-malignant cell populations which often appear to differ from the surrounding tissue both morphologically and genotypically.
- Such disorders may be associated with abnormal expression of a gene, for example, an increased level of expression, as well as expression of a mutant form of a gene, such that the normal function of the gene product is altered. Abnormal expression also includes inappropriate expression during the cell cycle or in an incorrect cell type. The above-described methods and vectors of the invention are useful in treating

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such malignancies of the various organ systems. For example, such therapy would achieve its therapeutic effect following introduction of the expression vector of the invention into cells having the proliferative disorder. Delivery of the vector can be achieved using any standard recombinant expression vectors. Various viral vectors 5 which can be utilized for gene therapy include, without limitation, adenovirus, adeno-associated virus, herpes virus, vaccinia, or an RNA virus such as a retrovirus.

The expression vectors of the invention are also useful in treating malignancies of the various organ systems, such as, for example, lung, breast, lymphoid, gastrointestinal, and genito-urinary tract as well as adenocarcinomas which 10 include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer, leukemia, breast cancer, non-small cell carcinoma of the lung, cancer of the small intestine, and cancer of the esophagus.

The methods and vectors of the invention are also useful in gene replacement therapy to reconstitute expression of a wild-type protein in cells 15 expressing a defective protein or, in the case of a heterozygote, a defective and a wild type protein. Therefore, the invention provides a method of silencing gene expression as described above and further includes administering a modified nucleic acid encoding a wild-type polypeptide corresponding to the gene product of the gene being suppressed. The replacement gene would then provide a protein with the 20 correct amino acid sequence utilizing codons with nucleotides that are not recognized by the sequences encoded by expression vectors of the invention. It is therefore possible both to silence the expression of a defective copy of a gene and to introduce a normal copy unaffected by the silencing expression vector.

There are also a number of inherited diseases in which defective genes 25 may be replaced. These include, without limitation, lysosomal storage diseases, such as those involving glucocerebrosidase, deficiencies in hypoxanthine phosphoribosyl transferase activity (the "Lesch-Nyhan" syndrome), and amyloid polyneuropathies (prealbumin).

In addition, in mammals, pathologic disorders that can be targeted using the methods

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and vectors of the invention include, but are not limited, to viral infections, inflammatory disorders, cardiovascular disease, cancers, genetic disorders, and autoimmune diseases.

The methods of the present invention are also useful for suppression of a dominant negative mutation. The term "dominant negative" refers to expression of a gene resulting in a gene product that actively interferes with the function of a wild type endogenous gene product (e.g., a protein). Thus, a mutant protein or dominantly active gene product, such as amyloid precursor protein that accumulates in Alzheimer's disease can be blocked. The dominant negative phenotype is conveyed by the expression of the mutant protein that interferes with the function of the normal protein. Such an effect is similar to the effect of dominance of one allele of a pair of alleles encoding homologous genes such that the phenotypic effect of the one allele exerts a deleterious controlling influence over the other allele.

Transgenic animals (e.g., transgenic non-human mammals) and plants can also be developed using the novel expression vectors and methods of the invention to identify the impact of increased or decreased gene expression on a particular pathway or phenotype. Standard protocols useful in producing such transgenic animals and plants are known in the art. Such protocols, for example, generally follow conventional techniques for introduction of expressible transgenes into animals such as mammals. Those of ordinary skill in the art will be familiar with these applications and will be readily able to apply the techniques in the context of the present invention. Accordingly, the expression vectors of the invention may be used to introduce DNA sequences into the germ line cells of non-humans to create transgenic animals. The preferred animal of the invention is a mouse. However, other non-humans of the invention include, but are not limited to, other rodents (e.g. rat, hamster), rabbits, chickens, sheep, goats, fish, pigs, cattle, and non-human primates.

Similarly, those skilled in the art will be familiar with methods for introducing transgenes into plants and will be able to apply the techniques in the

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context of the present invention. Accordingly, the expression vectors of the invention may be used to introduce DNA sequences into plant cells, and plant cells transformed with the plant expression vector can be regenerated, for example, from single cells, callus tissue, or leaf discs according to standard plant tissue culture techniques. Exemplary plants which are useful for generating the transgenic plants (or plant cells, plant tissues, plant organs, or plant parts) of the invention, include, without limitation, dicots and monocots, such as sugar cane, wheat, rice, maize, sugar beet, barley, grape, manioc, crucifer, mustard, potato, soybean, sorghum, cassava, banana, grape, oats, tomato, millet, coconut, orange, rye, cabbage, apple, eggplant, watermelon, canola, cotton, carrot, pepper, strawberry, peanut, legume, bean, pea, mango, and sunflower.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

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Claims

1. A method for silencing the expression of an endogenous gene in a cell, said method comprising overexpressing in said cell an isolated nucleic acid molecule of said endogenous gene and an antisense molecule comprising a nucleic acid molecule complementary to said nucleic acid molecule of said endogenous gene, wherein the overexpression of said nucleic acid molecule of said endogenous gene and said antisense molecule in said cell silences the expression of said endogenous gene.
2. The method of claim 1, wherein said isolated nucleic acid molecule of said endogenous gene generates an untranslatable RNA molecule.
3. The method of claim 1, wherein said cell is an animal cell, a mammalian cell, a cancer cell, or a plant cell.
4. The method of claim 1, wherein said isolated nucleic acid molecule of said endogenous gene comprises an exon.
- 15 5. The method of claim 4, wherein said exon is between 20-2,000 base pairs.
6. The method of claim 4, wherein overexpression of said exon and said antisense molecule silences the expression of a gene family or a specific member of a gene family.
- 20 7. The method of claim 1, wherein said endogenous gene encodes a metabolic enzyme, a structural protein, or a gene product that is associated with a disorder.

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8. The method of claim 7, wherein said disorder comprises cancer, tissue inflammation, or a dysfunction in a metabolic pathway.

9. The method of claim 1, wherein said endogenous gene encodes a gene product that when silenced confers on a plant an agronomically important trait.

5 10. The method of claim 1, wherein expression of said exon or antisense molecule is regulated by a tissue-specific or cell-specific expression control region or by a constitutive expression control region.

11. A method for silencing the expression of an endogenous gene in a cell, said method comprising expressing in said cell a vector comprising

10 (i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule comprising a proximal region and a distal region, said proximal region having substantial sequence identity to an endogenous gene of said cell;

15 wherein transcription of said DNA generates an RNA molecule having a genetically-engineered dsRNA stem-loop structure based on complementarity between nucleotides found in said distal region of said RNA molecule.

12. The method of claim 11, wherein said expression vector is overexpressed in said cell.

13. The method of claim 11, wherein said generated RNA molecule is 20 untranslatable.

14. The method of claim 11, wherein said cell is an animal cell, a mammalian cell, a cancer cell, or a plant cell..

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15. The method of claim 11, wherein the expression of said DNA molecule silences the expression of a gene family or a specific member of a gene family.

16. The method of claim 11, wherein said endogenous gene encodes a  
5 metabolic enzyme, a structural protein, or a gene product that is associated with a disorder.

17. The method of claim 16, wherein said disorder comprises cancer, tissue inflammation, or a dysfunction in a metabolic pathway.

18. The method of claim 11, wherein said endogenous gene encodes a  
10 gene product that when silenced confers on a plant an agronomically important trait.

19. The method of claim 11, wherein of said expression control region is a tissue-specific or cell-specific expression control region or a constitutive expression control region.

20. The method of claim 11, wherein said DNA comprises an exon.

15 21. The method of claim 20, wherein said exon is between 20-2,000 base pairs.

22. A gene silencing expression vector comprising  
(i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule comprising a proximal region and  
20 a distal region, said proximal region having substantial sequence identity  
to an endogenous gene of said cell;  
wherein transcription of said DNA generates an RNA molecule having a genetically-

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engineered dsRNA stem-loop structure based on complementarity between nucleotides found in said distal region of said RNA molecule.

23. The expression vector of claim 22, wherein said generated RNA molecule is untranslatable.

5 24. The expression vector of claim 22, wherein the expression of said DNA molecule silences the expression of a gene family or a specific member of a gene family.

10 25. The expression vector of claim 22, wherein said expression control region is a tissue-specific or cell-specific expression control region or is a constitutive expression control region.

26. The expression vector of claim 22, wherein said DNA comprises an exon.

27. The expression vector of claim 26, wherein said exon is between 20-2,000 base pairs.

15 28. A method for silencing the expression of an endogenous gene in a cell, said method comprising expressing in said cell a vector comprising  
(i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule having substantial sequence identity to an endogenous gene of said cell; wherein transcription of said DNA  
20 generates an RNA molecule having a dsRNA structure based on complementarity between nucleotides found in the 5' and 3' regions of said RNA molecule.

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29. A gene silencing expression vector comprising  
(i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule having substantial sequence  
identity to an endogenous gene of said cell; wherein transcription of said DNA  
5 generates an RNA molecule having a dsRNA based on complementarity between  
nucleotides found in the 5' and 3' regions of said RNA molecule.

30. A transgenic plant comprising the gene silencing expression vector  
of claim 22 or 29.

31. A transgenic plant comprising an overexpressed transgene encoding  
10 both an isolated nucleic acid molecule of an endogenous gene and an antisense  
molecule encoding a nucleic acid molecule complementary to the nucleic acid  
molecule of the endogenous gene.

32. A transgenic plant comprising an (i) overexpressed transgene  
encoding an isolated nucleic acid molecule of an endogenous gene and (ii) an  
15 overexpressed transgene encoding an antisense molecule encoding a nucleic acid  
molecule complementary to the nucleic acid molecule of the endogenous gene.

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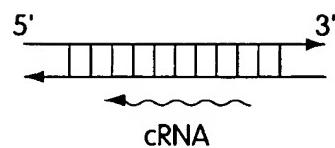


Fig. 1A

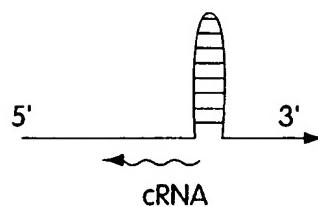


Fig. 1B

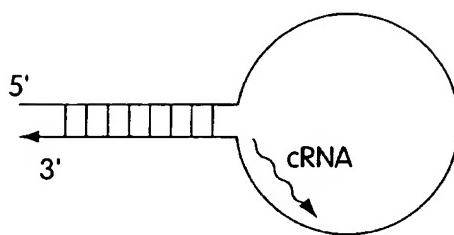


Fig. 1C

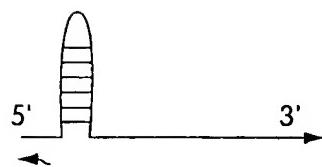
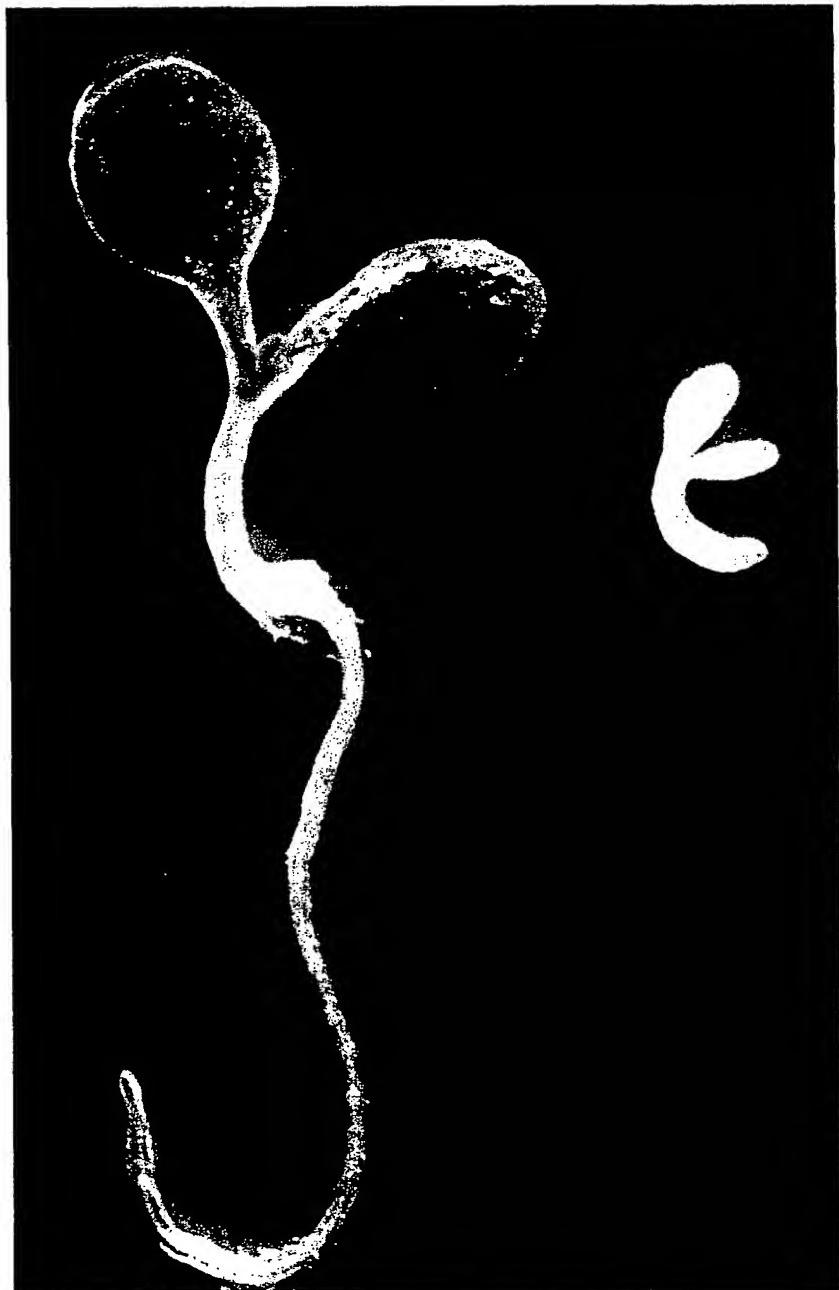


Fig. 1D

SUBSTITUTE SHEET (RULE 26)

2/2



A+ HXK      KNOCK-OUT      SENSE A+ HXK

Fig. 2

## INTERNATIONAL SEARCH REPORT

International application No.  
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## A. CLASSIFICATION OF SUBJECT MATTER

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US CL : 435/455, 419; 536/23.1, 24.5; 800/419, 295

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455, 419; 536/23.1, 24.5; 800/419, 295

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, BIOTECHNO, SCISEARCH, BIOSIS, EMBASE, CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOUGHERTY et al. Transgenes and Gene Supression: Telling Us Something New? Current Opinion in Cell Biology. 1995. Vol. 7, pages 399-405, see entire document.	1-32
Y	HAMADA et al. Co-Suppresion of the Hdrophobin Gene HCf-1 is Correlated with Antisense RNA Biosynthesis in Cladosporium fulvum. Mol. Gen. Genet. 1998. Vol. 259, pages 630-638, see entire document.	1-32
Y,P	JENSEN et al. Cosuppression of I Transposon Activity in Drosophila by I-Containing Sense and Antisense Transgenes. Genetics. 1999. Vol. 153, pages 1767-1774, see entire document.	1-32

<input checked="" type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
*A*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E*	earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O*	document referring to an oral disclosure, use, exhibition or other means	*&*	document member of the same patent family

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARTEA et al. Comparison of Sense and Antisense Methodologies for Modifying the Fatty Acid Composition of <i>Arabidopsis thalia</i> Oilseed. Plant Science. 1998. Vol. 136, pages 181-194, see entire document.	1-32

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WO 00/49035 A1

(54) Title: GENE SILENCING

(57) Abstract: Disclosed is a method for silencing the expression of an endogenous gene in a cell, the method involving overexpressing in the cell a nucleic acid molecule of the endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene, wherein the overexpression of the nucleic acid molecule of the endogenous gene and the antisense molecule in the cell silences the expression of the endogenous gene.

## GENE SILENCING

5

### Background of the Invention

This invention relates generally to the silencing of gene expression, as well as to expression vectors useful for silencing the expression of a target gene.

Cells manufacture proteins by transcribing genes encoded by DNA. In particular, one of the DNA strands of the gene is transcribed by an enzyme, RNA polymerase, to produce mRNA. The mRNA molecule has a base sequence that is complementary to that of the transcribed DNA strand. The mRNA is then processed by the removal of introns. The base sequence of the mRNA is next translated into the amino acid sequence of a protein molecule by means of the genetic code. This translation process requires many enzymes and a set of transfer RNA molecules, which align the amino acids according to the codon sequence. The translation of mRNA into protein occurs on ribosomes. The translated protein is often referred to as a gene product.

Developmental and physiological processes of cells and organisms require that the gene products necessary to carry out these processes are available in the appropriate amounts and at the appropriate times. For example, certain gene products must be present in all cells for many fundamental physiological processes to occur. Other gene products have tissue- or cell-specificity and are only necessary in certain cells or tissues. Some gene products are continuously present either in all cells or in certain cells or tissues. Still other gene products are required at different times during development.

Gene regulation, in both animals and plants, alters the quantity or quality or both of a gene product. These alterations can be used to ascertain the molecular activities of the normal gene product counterpart. Additionally, gene regulation can be used to manipulate cells and organisms at the genetic level. Gene regulation provides scientists with an expanded ability to study and treat disease processes. In particular, gene regulation techniques have proven especially useful in the elucidation

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and diagnosis of many diseases and abnormalities. Indeed, it is now possible to use gene regulation for therapeutic intervention and treatment at the genetic level. In addition, gene regulation can be used to identify and characterize genes involved in fundamental cellular and developmental processes of animals and plants. The

5 identification and characterization of these genes has previously been hampered by the fact that mutations in such genes are often lethal or are recessive in diploid organisms. Accordingly, the selective inactivation or regulation of genes has many potential uses.

Certain genes are regulated at the level of transcription. Transcriptional  
10 regulation is carried out either negatively (repressors) or positively (activators) by a protein factor. Specific protein factors regulate translation of specific mRNAs. It has also become evident that RNAs are involved in regulating the expression of specific genes.

Different approaches have attempted to regulate gene expression by  
15 selectively inactivating genes. For example, one approach to gene regulation is gene disruption. Gene disruption is accomplished using recombinant DNA techniques. It is generally a process of sequential elimination of the alleles for a particular gene. The alleles are eliminated by introducing a mutation into the gene, usually by homologous recombination, at the single cell stage of the organism, which renders the  
20 gene nonfunctional. Gene disruption is technically difficult and labor intensive. In addition, additional genes are sometimes unintentionally disrupted.

Another gene regulation approach involves the use of antisense RNA. Antisense RNA inhibition of specific mRNAs involves the use of DNA constructs that direct the transcription of an antisense RNA strand. For example, antisense DNA  
25 constructs have been prepared by flipping a gene fragment of interest and inserting this sequence between a promoter and a polyadenylation site in the inverse orientation. The RNA transcript obtained from this DNA molecule has a sequence complementary to a target mRNA. The antisense RNA anneals to the mRNA and disrupts normal processing or translation or both. Antisense constructs can be

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introduced into eukaryotic cells by standard transfection, transduction, or microinjection methods and function in both transient and stable transformation assays. Antisense transcripts complementary to the target gene mRNA specifically suppress gene activity.

5       Despite the availability of these and other methods for regulating gene expression, there exists a need for gene regulation methods that are capable of silencing or suppressing a specific gene product or multiple gene products.

#### Summary of the Invention

In one aspect, the invention features a method for silencing the expression 10 of a targeted gene (e.g., an endogenous gene) in a cell. The method generally involves overexpressing in the cell (e.g., an animal cell such as a mammalian cell, a cancer cell, or a plant cell) an isolated nucleic acid molecule of an endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene, wherein the overexpression of the 15 nucleic acid molecule of the endogenous gene and the antisense molecule in the cell silences the expression of the endogenous gene. Exemplary genes targeted for silencing include, without limitation, transcription regulatory factors, virally encoded proteins (e.g., human papilloma virus E6 and human immunodeficiency virus tat), structural proteins, metabolic or enzymatic proteins, cytokines, oncogenes, growth 20 factors (e.g., interleukins), gamma interferon, tumor necrosis factor, and granulocyte-macrophage-colony stimulating factor.

In preferred embodiments, the isolated nucleic acid molecule of an endogenous gene generates an untranslatable RNA molecule or a translatable RNA molecule that encodes a non-functional product. In other preferred embodiments, the 25 isolated nucleic acid molecule includes the coding region of the endogenous gene or a portion thereof. While in still other embodiments, the antisense molecule is complementary to a portion of the gene targeted for silencing.

Preferably, the isolated nucleic acid molecule of the endogenous gene used

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in the invention includes an exon (e.g., an exon that is between 20-2,000 base pairs).

In preferred embodiments, overexpression of the exon and the antisense molecule silences the expression of a gene family. In other preferred embodiments, overexpression of the exon and the antisense molecule silences the expression of a

5 specific member of the gene

family.

In yet other preferred embodiments, the expression of the isolated nucleic acid molecule, antisense molecule, or exon is controlled by an inducible expression control region, a tissue- or cell-specific expression control region, or by a (fully or 10 partially) constitutive expression control region.

In preferred embodiments, the endogenous gene selected for gene silencing encodes a protein, for example, a metabolic enzyme, a structural protein, or a gene product that is associated with a disorder (e.g., an autoimmune disease, cancer, tissue inflammation, or a dysfunction in a metabolic pathway). In still other preferred 15 embodiments, the endogenous gene encodes a gene product that when silenced confers on a plant an agronomically important trait (e.g., fruit ripening, senescence, male sterility, wounding response, disease resistance, or dessication tolerance).

In another aspect, the invention features a method for silencing the expression of an endogenous gene in a cell. The method generally involves 20 expressing in the cell a vector including (i) an expression control region functional in the cell; and (ii) an operably linked DNA molecule including a proximal region and a distal region, the proximal region having substantial sequence identity to an endogenous gene of the cell targeted for silencing, wherein transcription of the DNA generates an RNA molecule having a genetically-engineered dsRNA stem-loop 25 structure based on complementarity between nucleotides found in the distal region of the RNA molecule. In preferred embodiments, the expression vector is overexpressed in the cell.

In still another aspect, the invention features a gene silencing expression vector including (i) an expression control region functional in the cell; and (ii) an

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- operably linked DNA molecule including a proximal region and a distal region, the proximal region having substantial sequence identity to an endogenous gene of the cell targeted for silencing, wherein transcription of the DNA generates an RNA molecule having a genetically-engineered dsRNA stem-loop structure based on  
5 complementarity between nucleotides found in the distal region of the RNA molecule.

In another aspect, the invention features a method for silencing the expression of an endogenous gene in a cell. The method generally involves expressing in the cell a vector including: (i) an expression control region functional in the cell; and (ii) an operably linked DNA molecule having substantial sequence identity to an endogenous gene of the cell; wherein transcription of the DNA generates an RNA molecule that forms dsRNA structure based on complementarity between nucleotides found in the 5' and 3' regions of the RNA molecule.  
10

In yet another aspect, the invention features a gene silencing expression vector that includes: (i) an expression control region functional in the cell; and (ii) an operably linked DNA molecule having substantial sequence identity to an endogenous gene of the cell; wherein transcription of the DNA generates an RNA molecule that forms a dsRNA structure based on complementarity between nucleotides found in the 5' and 3' regions of the RNA molecule.  
15

In still another aspect, the invention features transgenic plants and non-human transgenic animals that overexpress in a cell (e.g., a non-human animal cell such as a mammalian cell, a cancer cell, or a plant cell) an isolated nucleic acid molecule of an endogenous gene and an antisense molecule including a nucleic acid molecule complementary to the nucleic acid molecule of the endogenous gene. The invention also features transgenic plants and non-human transgenic animals including  
20 the expression vector of the invention.  
25

As used herein, by "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

By "substantially identical" is meant a nucleic acid molecule exhibiting at

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least 40%, preferably 50%, more preferably 80%, and most preferably 90%, or even 95% contiguous sequence identity to a reference sequence. The length of comparison sequences will generally be at least 15-20 nucleotides, preferably at least 50 nucleotides, more preferably at least 75 nucleotides, and most preferably 100

5 nucleotides or more. Sequence identity is measured, for example, using standard sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705, Blast, BlastN, BlastP, BlastX, FastA, or PILEUP/Prettybox programs) set to standard parameters.

10 By "nucleic acid" or "nucleic acid molecule" is meant a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger expression vector. For example, nucleic acids can be assembled from cDNA fragments or from oligonucleotides to generate a synthetic gene which is capable of being expressed in a recombinant transcriptional unit,

15 generating transcripts that accumulate stably. Polynucleotide or nucleic acid molecules of the invention therefore include DNA, RNA, cDNA, or synthetic nucleic acid sequences.

By "isolated nucleic acid molecule" is meant a nucleic acid molecule (e.g., DNA) that is free of the genes which, in the naturally-occurring genome of the

20 organism from which the nucleic acid molecule is derived, flank the gene. The term therefore includes, for example, a gene or fragment thereof that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (for example, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease

25 digestion or a synthetic nucleic acid molecule) independent of other sequences. It also includes a recombinant nucleic acid molecule which is part of a hybrid gene encoding additional polypeptide sequence.

By "antisense molecule" is meant an RNA molecule that includes a nucleotide sequence that is complementary to a messenger RNA. In general, such an

antisense molecule will usually be at least 15 nucleotides, preferably, about 15-200 nucleotides, and, more preferably, 200-2,000 nucleotides in length. The antisense sequence may be complementary to all or a portion of the mRNA nucleotide sequence, and, as appreciated by those skilled in the art, the particular site or sites to which the antisense sequence binds as well as the length of the antisense sequence will vary, depending upon the degree of inhibition desired and the uniqueness of the antisense sequence. Antisense molecules may be constructed and expressed as described herein or as described, for example, in van der Krol et al., Gene 72, 45 (1988); Rodermel et al., Cell 55, 673 (1988); Mol et al., FEBS Lett. 268: 427 (1990); Weigel and Nilsson, Nature 377, 495 (1995); Cheung et al., Cell 82, 383 (1995); and U.S. Pat. No. 5,107,065.

By "obtained from" is meant isolated from or having the sequence of a naturally-occurring sequence (e.g., a cDNA, genomic DNA, synthetic DNA, or combination thereof).

By "expression control region" is meant any minimal sequence sufficient to direct transcription in a host cell (e.g., a plant or animal cell). Included in the invention are promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell-, tissue-, or organ-specific gene expression, or elements that are inducible by external signals or agents (for example, light-, pathogen-, wound-, stress-, or hormone-inducible elements or chemical inducers) or elements that are capable of cycling gene transcription; such elements may be located in the 5' or 3' regions of the native gene or engineered into a transgene construct.

By "exon" is meant any length of nucleic acid sequence, excluding intron sequences and excluding the non-coding regulatory sequences driving transcription. The exon sequence may be obtained in whole or in part from any source known in the art, including a plant, a fungus, an animal, a bacterial genome or episome, eukaryotic, nuclear or plasmid DNA, cDNA, viral DNA, or chemically synthesized DNA. If desired, an exon sequence of a target gene may contain one or more modifications in either the coding or the untranslated regions which affect the biological activity or the

chemical structure of the expression product, the rate of expression, or the manner of expression control. Such modifications include, but are not limited to, mutations, insertions, deletions, and substitutions of one or more nucleotides.

By "structural gene" is meant an uninterrupted sequence of a nucleic acid  
5 molecule, including one or more introns, bound by the appropriate splice junctions.

By "operably linked" is meant that a gene and a regulatory sequence(s) (e.g., an expression control region) are connected in such a way as to permit gene expression when the appropriate molecules (for example, transcriptional activator proteins) are bound to the regulatory sequence(s).

10 By "plant cell" is meant any self-propagating cell bounded by a semi-permeable membrane and containing a plastid. Such a cell also requires a cell wall if further propagation is desired. Plant cell, as used herein includes, without limitation, algae, cyanobacteria, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and  
15 microspores.

By "crucifer" is meant any plant that is classified within the Cruciferae family. The Cruciferae include many agricultural crops, including, without limitation, rape (for example, *Brassica campestris* and *Brassica napus*), broccoli, cabbage, brussel sprouts, radish, kale, Chinese kale, kohlrabi, cauliflower, turnip, rutabaga,  
20 mustard, horseradish, and *Arabidopsis*.

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an  
25 endogenous gene of the organism.

By "transgenic" is meant any cell which includes a nucleic acid molecule (e.g., a recombinant DNA molecule) which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants or non-human

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animals, and the DNA (transgene) is inserted by artifice into a nuclear or plastidic genome.

By "introducing into an animal or plant a nucleic acid" includes any number of ways of introducing a gene sequence into a eukaryotic cell that include, 5 without limitation, retroviruses, transfection, liposomes, *Agrobacterium*-mediated transformation, and biolistics, such that the introduced gene is maintained as an episome or incorporated into the genome of the cell, and is maintained and replicates as the cell divides and replicates.

By "gene therapy" is meant any therapy by which a biological deficit is 10 repaired by the introduction of a nucleic acid sequence that encodes a gene product capable of improving or delaying the disease.

By "target gene" is meant a gene that is pre-selected for gene silencing.

By "silencing" is meant that the expression of a gene is inhibited, suppressed, or decreased. Preferably, a gene is considered silenced when its 15 expression is inhibited, suppressed, or decreased, for example, by at least 50% of the normal expression level, preferably by at least 75%, and more preferably by at least 90%.

By "genetically-engineered" is meant to splice, modify, or alter a nucleic acid molecule using standard recombinant molecular techniques or methodologies. A 20 DNA molecule, for example, may be modified or altered to include site specific mutations into one or more coding and/or noncoding regions which result, upon transcription, in the production of an RNA molecule having a stem-loop structure. Such techniques may also be used to engineer a mutation, including, but not limited to an insertion, deletion, or substitution of one or more nucleotides of a nucleic acid 25 molecule (e.g., a DNA molecule).

By "agronomically important trait" is meant a trait or characteristic found in a plant that contributes to its economic value. Such traits include, without limitation, stress tolerance or resistance or both, as exemplified by resistance or tolerance to drought, heat, chilling, freezing, excessive moisture, salt stress; oxidative

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stress; increased yields; food content and makeup; physical appearance; male sterility; starch properties; quantity and quality of secondary compounds; sugar quantity and quality; oil quantity and quality; and protein quantity and quality. Accordingly, one may desire to silence one or more genes involved in a regulatory or biosynthetic 5 pathway as a means of conferring any such desirable agronomically important trait or traits on a plant.

The invention is useful for modifying the activity of an enzyme; to provide for the specific or preferential expression of an allele or one or members of a multigene family; or for the expression of a particular isozyme. In addition, the 10 invention is useful for modulating metabolic pathways or plant development.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Detailed Description

The drawing will first be described.

15 **Drawing**

Figure 1 shows exemplary mechanisms for utilizing dsRNA-based gene silencing for the synthesis of cRNA (complementary RNA). The cRNA is typically not capped and does not have include a polyA<sup>+</sup> tail, which enhances the degradation of the mRNA encoded by a preselected target gene, and for its transport through 20 phloem and plasmodesmata of plants.

Figure 2 is a photograph showing the effects of overexpressing sense and antisense *AtHXK1* genes in transgenic *Arabidopsis* seedlings germinated on 7% glucose/MS. The transgenic seedling expressing both sense and antisense constructs is shown on the left; the seedling expressing sense *AtHXK1* is shown on the right.

25 **Gene Silencing Expression Vectors**

The present invention includes novel nucleic acid expression vectors which provide a general means for silencing the expression of pre-selected target

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genes. The expression vectors of the invention generally include an expression control region and an isolated nucleic acid molecule (e.g., a DNA molecule) having substantial sequence identity to a gene whose expression is selected to be silenced (e.g., an endogenous gene). Upon transcription, the DNA molecule generates an RNA 5 molecule. A portion of this generated RNA molecule then folds into a double-stranded (ds) RNA stem-loop or a region of dsRNA. Also included in the invention is a method of silencing gene expression utilizing the expression vector of the invention.

Nucleic acid molecules utilized in the invention can be obtained by several methods. Sequences for specific genes or stem-loop structures, for example, can be 10 obtained from published sequences and can also be found in the GenBank computer database (Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>), or may be determined after isolation using standard techniques. Nucleic acids can then be chemically synthesized, if desired, by standard methods.

#### Double-Stranded RNA Stem-Loops

15 As is discussed above, the expression vector of the invention which is useful for silencing the expression of an endogenous gene or virtually any other targeted gene includes a DNA molecule that, upon transcription, generates an RNA molecule having a stem-loop structure. Preferably, the stem-loop structure, within the generated RNA molecule, is positioned distally relative to the region of the RNA 20 molecule encoding the sequence of the gene selected for silencing. While not wanting to be bound by a particular theory, it is believed that the stem-loop structure provides a binding site or template for a dsRNA-dependent RNA polymerase. dsRNA-dependent RNA polymerase in turn synthesizes a cRNA based on the RNA template of the targeted gene. Exemplary strategies for designing expression constructs that 25 express RNA molecules useful for the synthesis of cRNA are shown in Fig. 1. The synthesis of the cRNA is thought to promote RNA degradation and concomitant silencing of the target gene.

The stem-loop structure refers to a nucleic acid structure that folds preferably into a hairpin form. Stem-loop structures used in the expression vectors of

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the invention are typically unmodified, naturally-occurring structures, and are readily engineered for incorporation into the expression vector using standard cloning techniques. Alternatively, standard methods may be employed to synthesize stem-loop structures that mimic the naturally-occurring structures. Such genetically  
5 engineered stem-loop structures, like naturally-occurring stem-loop structures, can then be integrated into the expression vector of the invention. It will be understood that the folding pattern of the stem-loop structure is not compromised by alterations in the nucleic acid sequence of the naturally-occurring molecule. For example, it is understood that alterations which include, but are not limited to, mutations, insertions,  
10 deletions, and substitutions of one or more nucleotides can be made within the sequence of the stem-loop, as long as the hairpin formation of the stem-loop is maintained. Hairpin structures useful in the invention are preferably 100-200 base pairs or more in length.

In addition, a DNA molecule may be genetically engineered to express an  
15 RNA molecule that forms a dsRNA structure based on complementarity found between nucleotides in its 5' and 3' regions (Fig. 1). Preferably, the dsRNA structure is approximately 20-50 bp, or is of a size sufficient to provide a template for a dsRNA-dependent RNA polymerase. The remaining non-complementary region of the RNA molecule has substantial identity to a sequence encoding a preselected target  
20 gene.

dsRNA-dependent RNA polymerase

Constructs expressing a dsRNA-dependent RNA polymerase may also be introduced in a cell according to standard methods known in the art. Exemplary polymerases are described in Schiebel et al. (Plant Cell 10: 2087, 1998) and Cogoni et  
25 al. (Nature 399: 166, 1999). Expression of a dsRNA-dependent RNA polymerase facilitates RNA degradation and, consequently, gene silencing. For example, if a cell does not express an endogenous dsRNA-dependent RNA polymerase or expresses an insufficient level of the enzyme, a heterologous dsRNA-dependent RNA polymerase

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from any number of organisms (e.g., a plant) may be introduced to promote the generation of sequence-specific cRNAs.

Target Sequences

The expression vector of the invention also includes an isolated nucleic acid molecule (e.g., a DNA molecule) that encodes an RNA molecule corresponding to a pre-selected target gene (e.g., an endogenous gene of a cell) whose expression is to be silenced. Thus, the nucleic acid molecule once expressed in the cell silences the expression of virtually any target gene. For example, if hexokinase gene expression is to be silenced in a cell, then the expression vector of the invention incorporates a DNA molecule that is substantially identical to an endogenous hexokinase gene of the cell.

The DNA molecule utilized in the expression vector of the invention is generally obtained directly from the target gene that is to be silenced and differs, as is discussed above, primarily by the inclusion of a region which, upon transcription, folds into a dsRNA stem-loop structure. Furthermore, the DNA molecule, if desired, may be chemically synthesized. The DNA molecule encoding the RNA molecule has generally over 90% identity to the target gene to be silenced. However, DNA molecules having less than 90% identity to the target gene sequence may also be utilized; for example, a DNA molecule encoding an RNA molecule will also be effective when it shares at least 80% sequence identity with the target gene to be silenced.

While a DNA molecule may encode an RNA molecule corresponding to the full length sequence of the target gene, substantially less than the full length of the target gene can be used. For example, a DNA molecule corresponding to a portion of the target gene may also be utilized. Accordingly, relatively short regions of the target gene may be used to engineer a DNA molecule depending on the size of the target gene to be silenced.

In particular examples, in some applications, the DNA molecule of the expression vector may be 15-200 nucleotides in length, although longer sequences,

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such as 100-250 nucleotides may also be utilized. In such applications where such shorter sequences (e.g., less than 200 nucleotides) are utilized, the introduced DNA molecule will share a high degree of identity with the target gene sequence, being preferably at least 80% or even 90% identical. In other applications, a sequence of  
5 greater than 500 nucleotides may be used, again depending on the size of the target gene to be silenced.

Thus, the DNA molecule employed in the expression vector of the invention generally includes one contiguous nucleic acid sequence of at least 15-20 nucleotides in length that has at least 80% identity to a sequence found in the target  
10 gene to be silenced. In other applications, the DNA molecule includes at least one contiguous nucleic acid sequence of at least 100 nucleotides in length that has at least 80% identity to the target gene. And, in still other applications, the DNA molecule includes at least one contiguous nucleic acid sequence of at least 250 nucleotides in length that has at least 85% identity to the target gene.

15 Because a full length sequence is unnecessary, the invention is not limited to the silencing of individual target genes. It is also possible to silence the expression of multiple genes using a single introduced DNA molecule that contains sequences corresponding to several genes (e.g., members of a multigene family) that are expressed in the target cell. Alternatively, it is possible to silence the expression of  
20 specific members of a multigene family using a single introduced DNA molecule that contains a gene-specific sequence (e.g., sequences typically found in the 3' untranslated regions of a mRNA transcript) corresponding to the specific gene family member to be silenced in the target cell.

Once a target gene to be silenced is identified, a DNA molecule having  
25 substantial identity to the target gene sequence is manipulated to render the RNA molecule encoded by the DNA molecule to include a stem-loop structure, using standard cloning methodologies. Furthermore, the proximal region of the DNA molecule itself may be tailored and manipulated using standard *in vitro* mutagenesis (e.g., using those methods described below) to modify the molecule so that upon

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transcription stable dsRNA stem-loop structure formation is facilitated.

Additionally, the RNA molecule encoded by the DNA molecule may be rendered untranslatable by employing standard methods. For example, the translation initiation codon (e.g., an ATG) can be removed from the DNA sequence.

- 5 Alternatively, additional translational initiation codons may be introduced into the DNA molecule. Furthermore, the reading frame of the DNA sequence can be displaced by the addition of one or more bases. Stop codons may also be introduced after initiation codons. Many standard DNA mutagenesis techniques are available to introduce such modifications into the DNA molecule including, for example, M13 primer mutagenesis. Details of such techniques are provided in Sambrook et al.,  
10 *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York. (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, NY (1998).

Expression Control Regions

- 15 A wide range of expression control regions may be utilized in the expression vector of the invention, including viral, plant, or mammalian promoters. Additionally, cell- or tissue-specific promoters can, if desired, be used to trigger expression of a nucleic acid molecule in specific cell populations.

Mammalian and viral promoters suitable for use in the present invention  
20 are available in the art. The choice of a particular promoter as an expression control region of the invention, is dictated by the spatial and temporal pattern of expression that is desired for the expression of the target gene encoded by the DNA molecule of the expression vector, and ultimately for the location where the target gene is to be silenced. Expression control regions of the invention therefore include eukaryotically derived promoters which predominantly direct expression in, for example, the reproductive system (e.g., breast, ovary, testes); the musculoskeletal system (e.g., muscle or joint tissue); the cardiovascular system (e.g., capillaries or heart); the respiratory system (e.g., lung or nasal passages); the urological system (e.g., kidney or bladder); the gastrointestinal system (e.g., pancreas, liver, or intestines); the immune

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system (e.g., thymus, spleen, or circulating immunological cells); the endocrine system (e.g., pituitary, gonads, and thyroid); the nervous system (e.g., neurons); or the hematopoietic system (e.g., bone marrow and peripheral blood). Further, promoters of the invention include, but are not limited to, the elastase promoter (expression in 5 pancreatic acinar cells); the alpha-A-crystallin promoter (expression in the eye lens tissue); the insulin promoter (expression in the pancreatic beta cells), and the albumin promoter regions.

Additionally, non-eukaryotically-derived promoters such as virally-derived and prokaryotically-derived promoters are included in the present invention. Such 10 promoters include, but are not limited, to the mouse mammary tumor virus promotor (MMTV), SV40 early region promoter, Rous sarcoma virus (RSV) promoter, or cytomegalovirus (CMV) promoter, which direct expression of viral or host genes in specific tissues and in many cell types.

In addition to the above-described expression control regions, a number of 15 gene promoters which are active in plant cells have been described in the literature. An example of a useful plant promoter according to the invention is a caulimovirus promoter, for example, a cauliflower mosaic virus (CaMV) promoter. These promoters confer high levels of expression in most plant tissues, and the activity of these promoters is not dependent on virally encoded proteins. CaMV is a source for 20 both the 35S and 19S promoters. In most tissues of transgenic plants, the CaMV 35S promoter is a strong promoter (see, e.g., Odell et al., Nature 313:810, 1985). The CaMV promoter is also highly active in monocots (see, e.g., Dekeyser et al., Plant Cell 2:591, 1990; Terada and Shimamoto, Mol. Gen. Genet. 220:389, 1990). Moreover, activity of this promoter can be further increased (i.e., between 2-10 fold) 25 by duplication of the CaMV 35S promoter (see e.g., Kay et al., Science 236:1299, 1987; Ow et al., Proc. Natl. Acad. Sci., U.S.A. 84:4870, 1987; and Fang et al., Plant Cell 1:141, 1989). In addition, a minimal 35S promoter may also be used as is described herein.

Other useful plant promoters include, without limitation, the nopaline

synthase promoter (An et al., Plant Physiol. 88:547, 1988) and the octopine synthase promoter (Fromm et al., Plant Cell 1:977, 1989). Exemplary monocot promoters include, without limitation, commelina yellow mottle promoter, sugar cane badna virus promoter, rice tungro baciliform virus promoter, maize streak virus element, and 5 wheat dwarf virus promoter.

For certain applications in plants, it may be desirable to express the DNA molecule of the expression construct in an appropriate tissue, at an appropriate level, or at an appropriate developmental time. For these purposes, there are an assortment of gene promoters, each with its own distinct characteristics embodied in its 10 regulatory sequences, shown to be regulated in response to the environment, hormones, and/or developmental cues. These include gene promoters that are responsible for heat-regulated gene expression (see, e.g., Callis et al., Plant Physiol. 88:965, 1988; Takahashi and Komeda, Mol. Gen. Genet. 219:365, 1989; and 15 Takahashi et al., Plant J. 2:751, 1992), light-regulated gene expression (e.g., the *Arabidopsis Cab2* photosynthetic, leaf specific promoter described by Mitra et al., Plant Mol. Biol. 12: 169-179, 1989; the pea *rbcS-3A* described by Kuhlemeier et al., Plant Cell 1:471, 1989; the maize *rbcS* promoter described by Schäffner and Sheen, Plant Cell 3:997, 1991; or the chlorophyll a/b-binding protein gene found in pea 20 described by Simpson et al., EMBO J. 4:2723, 1985), hormone-regulated gene expression (for example, the abscisic acid (ABA) responsive sequences from the *E<sub>m</sub>* gene of wheat described by Marcotte et al., Plant Cell 1:969, 1989; the ABA-inducible HVA1 and HVA22, and rd29A promoters described for barley and 25 *Arabidopsis* by Straub et al., Plant Cell 6:617, 1994, Shen et al., Plant Cell 7:295, 1995; and wound-induced gene expression (for example, of *wunI* described by Siebertz et al., Plant Cell 1:961, 1989), organ-specific gene expression (for example, of the tuber-specific storage protein gene described by Roshal et al., EMBO J. 6:1155, 1987; the 23-kDa zein gene from maize described by Schernthaner et al., EMBO J. 7:1249, 1988; or the French bean  $\beta$ -phaseolin gene described by Bustos et al., Plant Cell 1:839, 1989; the vegetative storage protein promoter (soybean *vspB*) described

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by Sadka et al (Plant Cell 6: 737-749, 1994)), cycling promoters (e.g., the *Arabidopsis* cdc2a promoter described by Hemerly et al., Proc Natl Acad Sci USA 89: 3295-3299, 1992), senescence-specific promoters (e.g., the *Arabidopsis* SAG12 promoter described by Gan et al, Science: 270, 1986-1988, 1995), seed-specific promoters (for example, endosperm-specific or embryo-specific promoters), meristem-specific promoters (for example, the *Tch4* promoter described by Xu et al., Plant Cell 7:1555-67, 1995), or pathogen-inducible promoters (for example, PR-1 or  $\beta$ -1,3 glucanase promoters).

Transformation

Upon construction of the expression vector of the invention, several standard methods are available for introduction of the vector into a host cell (e.g., a plant or an animal). The method of transformation is not critical to the invention. Any method which provides for efficient transformation may be employed. As newer methods are available to transform host cells, they may be directly applied.

15 Gene Silencing By Overexpression of Sense and Antisense RNAs

Gene silencing is also accomplished by the simultaneous overexpression of sense and antisense RNA transcripts having substantial identity to a gene targeted for regulation. The invention therefore includes a method of silencing a gene by introducing into a cell a sense DNA construct of the targeted gene and its antisense counterpart. Using this simple approach, virtually any gene (e.g., an endogenous gene) can be regulated by the methods of the invention.

Expression vectors designed to overexpress sense and antisense transcripts of a gene targeted for regulation are constructed according to standard methods (e.g., those described herein). For example, a eukaryotic expression vector system (e.g., a cell- or tissue-specific vector) may be used to express a DNA molecule (in the sense or antisense orientation on either the same or individual vectors). Appropriate gene sequences are introduced into a plasmid or other vector, which construct is then used to transform living cells according to standard methods. Constructs containing the

entire open reading frame, inserted as a translatable or untranslatable transcript, may be used. Alternatively, portions of a DNA molecule, may be inserted. One of skill in the art will readily be able to determine which sequences to use as the appropriate sense and antisense nucleic acid construct.

5       The gene silencing effect on a targeted gene using the methods of the invention is typically monitored using standard methods. For example, the effectiveness of the simultaneous overexpression of sense and antisense genes in silencing gene expression can be determined phenotypically or by standard Northern blot analysis or immunohistochemically. Other standard nucleic acid detection  
10 techniques or alternatively immunodiagnostic techniques known to those of skill in the art (e.g., Western or Northwestern blot analysis) may be employed. Typically, a gene is considered silenced when its expression is suppressed or inhibited for example, by at least 50% of the normal expression, preferably by at least 75%, and more preferably by at least 90%.

15       The following example is provided for the purpose of illustrating the invention, and should not be construed as limiting.

To demonstrate the application of the invention, a transgenic plant was generated which overexpressed both a sense transcript and an antisense transcript (~ 1,500 base pairs (bp)) complementary to the sense transcript. Using the hexokinase 20 (HXK) *AtHXK1* gene of the crucifer *Arabidopsis*, sense and antisense expression constructs were generated as described in Sheen et al., W097/35965. Gene constructs overexpressing either the sense or antisense RNAs of the coding sequences from the HXK1 gene were introduced into separate *Arabidopsis* lines. These lines were then crossed to produce plants overexpressing both sense and antisense RNAs. Plants resulting from the crosses displayed a stronger glucose insensitive phenotype than control plants. The HXK mutant was easily identified based on its ability to develop roots and expanded green cotyledons (Fig. 2). Additionally, these new HXK mutants were observed to display a stronger glucose insensitive phenotype than the null 25 *AtHXK1* mutant or the antisense *AtHXK1* transgenic plant. The glucose insensitive *AtHXK1* mutant or the antisense *AtHXK1* transgenic plant. The glucose insensitive

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phenotype was taken as an indication that the expression of the HXK gene was silenced or knocked out by formation of double-stranded (ds) HXK RNA and resultant silencing of the endogenous *Arabidopsis* HXK gene. Finally, although plants overexpressing sense *AtHXK1* were found to have 20-50 times more AtHXK1 protein, no AtHXK1 protein was observed in plants overexpressing both the sense and antisense *AtHXK1* constructs. Similar results could be obtained by introducing sense and antisense DNA constructs in one expression vector, and introducing such an expression vector into a plant using standard methods.

In another example of simultaneously expressing sense and antisense constructs in the same plant, transgenic *Arabidopsis* plants expressing a full-length CDPK1 (calcium dependent protein kinase 1) cDNA were first generated as described in Sheen, WO 98/26045, using kanamycin as a selectable marker. Plants overexpressing the CDPK1 cDNA were observed as drought resistant. A homozygous sense CDPK1 T3 line was then generated, and two different antisense CDPK1 constructs (~ 250 bp and 700 bp), that shared identity between the CDPK1 and CDPK1a genes, were separately introduced into this line using standard *Agrobacterium* mediated plant cell transformation. Transgenic plants expressing both the sense and antisense constructs were then identified using bar and kanamycin selection. In plants expressing both sense and antisense constructs, the drought resistance phenotype was eliminated, and these plants were further found to be more sensitive to drought conditions than wild type plants. In addition, plants expressing both sense and antisense constructs were observed to have late flowering and aerial leaf phenotypes.

25 Uses

The invention described herein is useful for a variety of agricultural, horticultural, and medicinal purposes. For example, the expression vector(s) and methods described above are useful for silencing the expression of virtually any targeted gene or a particular metabolic pathway.

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- In the agricultural and horticultural fields, for example, the invention is particularly useful for silencing the expression of a variety of genes including, without limitation, genes associated with, for example, agronomically important traits, such as the synthesis or metabolism of peptides, proteins, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, fragrances, toxins, carotenoid pigments, hormones, cell wall polymers, gene regulatory molecules, flavonoids, storage proteins, phenolic acids, coumarins, alkaloids, quinones, lignins, glucosinolates, tannins, aliphatic amines, celluloses, polysaccharides, glycoproteins, and glycolipids.
- For instance, an alteration in the production of fatty acids or lipids can be engineered (and fatty acid composition of, e.g., an oil-producing plant thus altered) by blocking synthesis of a specific chain elongation or desaturation enzyme. Also, the synthesis of starch and sugars can be reduced (and sugar content of, e.g., an edible plant thus altered) by blocking enzymes required for starch and carbohydrate synthesis. In addition, ethylene production can be blocked to delay senescence. Similarly, fragrant molecules can be released from cells (thus altering the scent characteristics of, e.g., ornamental flowers) by blocking the enzymes responsible for glycosylation of such molecules. In addition, male-sterile plants may be engineered by silencing genes responsible for the formation of the male gametes.
- In medicine, the invention provides a method of gene therapy for the treatment of cell proliferative or immunologic disorders and diseases such as those that are mediated by various overexpressed proteins. Exemplary cell-proliferative disorders include malignant as well as non-malignant cell populations which often appear to differ from the surrounding tissue both morphologically and genotypically. Such disorders may be associated with abnormal expression of a gene, for example, an increased level of expression, as well as expression of a mutant form of a gene, such that the normal function of the gene product is altered. Abnormal expression also includes inappropriate expression during the cell cycle or in an incorrect cell type. The above-described methods and vectors of the invention are useful in treating

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such malignancies of the various organ systems. For example, such therapy would achieve its therapeutic effect following introduction of the expression vector of the invention into cells having the proliferative disorder. Delivery of the vector can be achieved using any standard recombinant expression vectors. Various viral vectors  
5 which can be utilized for gene therapy include, without limitation, adenovirus, adeno-associated virus, herpes virus, vaccinia, or an RNA virus such as a retrovirus.

The expression vectors of the invention are also useful in treating malignancies of the various organ systems, such as, for example, lung, breast, lymphoid, gastrointestinal, and genito-urinary tract as well as adenocarcinomas which  
10 include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer, leukemia, breast cancer, non-small cell carcinoma of the lung, cancer of the small intestine, and cancer of the esophagus.

The methods and vectors of the invention are also useful in gene replacement therapy to reconstitute expression of a wild-type protein in cells  
15 expressing a defective protein or, in the case of a heterozygote, a defective and a wild type protein. Therefore, the invention provides a method of silencing gene expression as described above and further includes administering a modified nucleic acid encoding a wild-type polypeptide corresponding to the gene product of the gene being suppressed. The replacement gene would then provide a protein with the  
20 correct amino acid sequence utilizing codons with nucleotides that are not recognized by the sequences encoded by expression vectors of the invention. It is therefore possible both to silence the expression of a defective copy of a gene and to introduce a normal copy unaffected by the silencing expression vector.

There are also a number of inherited diseases in which defective genes  
25 may be replaced. These include, without limitation, lysosomal storage diseases, such as those involving glucocerebrosidase, deficiencies in hypoxanthine phosphoribosyl transferase activity (the "Lesch-Nyhan" syndrome), and amyloid polyneuropathies (prealbumin).

In addition, in mammals, pathologic disorders that can be targeted using the methods

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and vectors of the invention include, but are not limited, to viral infections, inflammatory disorders, cardiovascular disease, cancers, genetic disorders, and autoimmune diseases.

The methods of the present invention are also useful for suppression of a  
5 dominant negative mutation. The term "dominant negative" refers to expression of a gene resulting in a gene product that actively interferes with the function of a wild type endogenous gene product (e.g., a protein). Thus, a mutant protein or dominantly active

gene product, such as amyloid precursor protein that accumulates in Alzheimer's  
10 disease can be blocked. The dominant negative phenotype is conveyed by the expression of the mutant protein that interferes with the function of the normal protein. Such an effect is similar to the effect of dominance of one allele of a pair of alleles encoding homologous genes such that the phenotypic effect of the one allele exerts a deleterious controlling influence over the other allele.

15 Transgenic animals (e.g., transgenic non-human mammals) and plants can also be developed using the novel expression vectors and methods of the invention to identify the impact of increased or decreased gene expression on a particular pathway or phenotype. Standard protocols useful in producing such transgenic animals and plants are known in the art. Such protocols, for example, generally follow  
20 conventional techniques for introduction of expressible transgenes into animals such as mammals. Those of ordinary skill in the art will be familiar with these applications and will be readily able to apply the techniques in the context of the present invention. Accordingly, the expression vectors of the invention may be used to introduce DNA sequences into the germ line cells of non-humans to create transgenic animals. The  
25 preferred animal of the invention is a mouse. However, other non-humans of the invention include, but are not limited to, other rodents (e.g. rat, hamster), rabbits, chickens, sheep, goats, fish, pigs, cattle, and non-human primates.

Similarly, those skilled in the art will be familiar with methods for introducing transgenes into plants and will be able to apply the techniques in the

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context of the present invention. Accordingly, the expression vectors of the invention may be used to introduce DNA sequences into plants cells, and plant cells transformed with the plant expression vector can be regenerated, for example, from single cells, callus tissue, or leaf discs according to standard plant tissue culture techniques. Exemplary plants which are useful for generating the transgenic plants (or plant cells, plant tissues, plant organs, or plant parts) of the invention, include, without limitation, dicots and monocots, such as sugar cane, wheat, rice, maize, sugar beet, barley, grape, manioc, crucifer, mustard, potato, soybean, sorghum, cassava, banana, grape, oats, tomato, millet, coconut, orange, rye, cabbage, apple, eggplant, watermelon, canola, cotton, carrot, pepper, strawberry, peanut, legume, bean, pea, mango, and sunflower.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

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Claims

1. A method for silencing the expression of an endogenous gene in a cell, said method comprising overexpressing in said cell an isolated nucleic acid molecule of said endogenous gene and an antisense molecule comprising a nucleic acid molecule complementary to said nucleic acid molecule of said endogenous gene,  
5 wherein the overexpression of said nucleic acid molecule of said endogenous gene and said antisense molecule in said cell silences the expression of said endogenous gene.
2. The method of claim 1, wherein said isolated nucleic acid molecule of  
10 said endogenous gene generates an untranslatable RNA molecule.
3. The method of claim 1, wherein said cell is an animal cell, a mammalian cell, a cancer cell, or a plant cell.
4. The method of claim 1, wherein said isolated nucleic acid molecule of said endogenous gene comprises an exon.  
15
5. The method of claim 4, wherein said exon is between 20-2,000 base pairs.
6. The method of claim 4, wherein overexpression of said exon and said antisense molecule silences the expression of a gene family or a specific member of a gene family.
- 20
7. The method of claim 1, wherein said endogenous gene encodes a metabolic enzyme, a structural protein, or a gene product that is associated with a disorder.

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8. The method of claim 7, wherein said disorder comprises cancer, tissue inflammation, or a dysfunction in a metabolic pathway.

9. The method of claim 1, wherein said endogenous gene encodes a gene product that when silenced confers on a plant an agronomically important trait.

5 10. The method of claim 1, wherein expression of said exon or antisense molecule is regulated by a tissue-specific or cell-specific expression control region or by a constitutive expression control region.

11. A method for silencing the expression of an endogenous gene in a cell, said method comprising expressing in said cell a vector comprising

10 (i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule comprising a proximal region and a distal region, said proximal region having substantial sequence identity to an endogenous gene of said cell;

15 wherein transcription of said DNA generates an RNA molecule having a genetically-engineered dsRNA stem-loop structure based on complementarity between nucleotides found in said distal region of said RNA molecule.

12. The method of claim 11, wherein said expression vector is overexpressed in said cell.

13. The method of claim 11, wherein said generated RNA molecule is 20 untranslatable.

14. The method of claim 11, wherein said cell is an animal cell, a mammalian cell, a cancer cell, or a plant cell.

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15. The method of claim 11, wherein the expression of said DNA molecule silences the expression of a gene family or a specific member of a gene family.

16. The method of claim 11, wherein said endogenous gene encodes a  
5 metabolic enzyme, a structural protein, or a gene product that is associated with a disorder.

17. The method of claim 16, wherein said disorder comprises cancer, tissue inflammation, or a dysfunction in a metabolic pathway.

18. The method of claim 11, wherein said endogenous gene encodes a  
10 gene product that when silenced confers on a plant an agronomically important trait.

19. The method of claim 11, wherein of said expression control region is a tissue-specific or cell-specific expression control region or a constitutive expression control region.

20. The method of claim 11, wherein said DNA comprises an exon.

15 21. The method of claim 20, wherein said exon is between 20-2,000 base pairs.

22. A gene silencing expression vector comprising  
20 (i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule comprising a proximal region and a distal region, said proximal region having substantial sequence identity to an endogenous gene of said cell;  
wherein transcription of said DNA generates an RNA molecule having a genetically-

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engineered dsRNA stem-loop structure based on complementarity between nucleotides found in said distal region of said RNA molecule.

23. The expression vector of claim 22, wherein said generated RNA molecule is untranslatable.

5 24. The expression vector of claim 22, wherein the expression of said DNA molecule silences the expression of a gene family or a specific member of a gene family.

10 25. The expression vector of claim 22, wherein said expression control region is a tissue-specific or cell-specific expression control region or is a constitutive expression control region.

26. The expression vector of claim 22, wherein said DNA comprises an exon.

27. The expression vector of claim 26, wherein said exon is between 20-2,000 base pairs.

15 28. A method for silencing the expression of an endogenous gene in a cell, said method comprising expressing in said cell a vector comprising  
(i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule having substantial sequence identity to an endogenous gene of said cell; wherein transcription of said DNA  
20 generates an RNA molecule having a dsRNA structure based on complementarity between nucleotides found in the 5' and 3' regions of said RNA molecule.

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29. A gene silencing expression vector comprising  
(i) an expression control region functional in said cell; and  
(ii) an operably linked DNA molecule having substantial sequence  
identity to an endogenous gene of said cell; wherein transcription of said DNA  
5 generates an RNA molecule having a dsRNA based on complementarity between  
nucleotides found in the 5' and 3' regions of said RNA molecule.

30. A transgenic plant comprising the gene silencing expression vector  
of claim 22 or 29.

31. A transgenic plant comprising an overexpressed transgene encoding  
10 both an isolated nucleic acid molecule of an endogenous gene and an antisense  
molecule encoding a nucleic acid molecule complementary to the nucleic acid  
molecule of the endogenous gene.

32. A transgenic plant comprising an (i) overexpressed transgene  
encoding an isolated nucleic acid molecule of an endogenous gene and (ii) an  
15 overexpressed transgene encoding an antisense molecule encoding a nucleic acid  
molecule complementary to the nucleic acid molecule of the endogenous gene.

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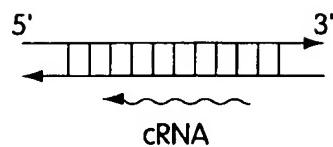


Fig. 1A

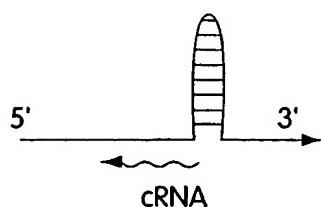


Fig. 1B

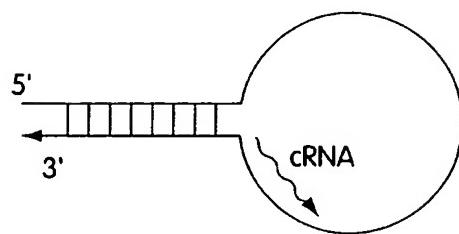


Fig. 1C

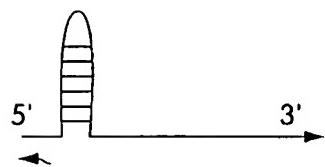
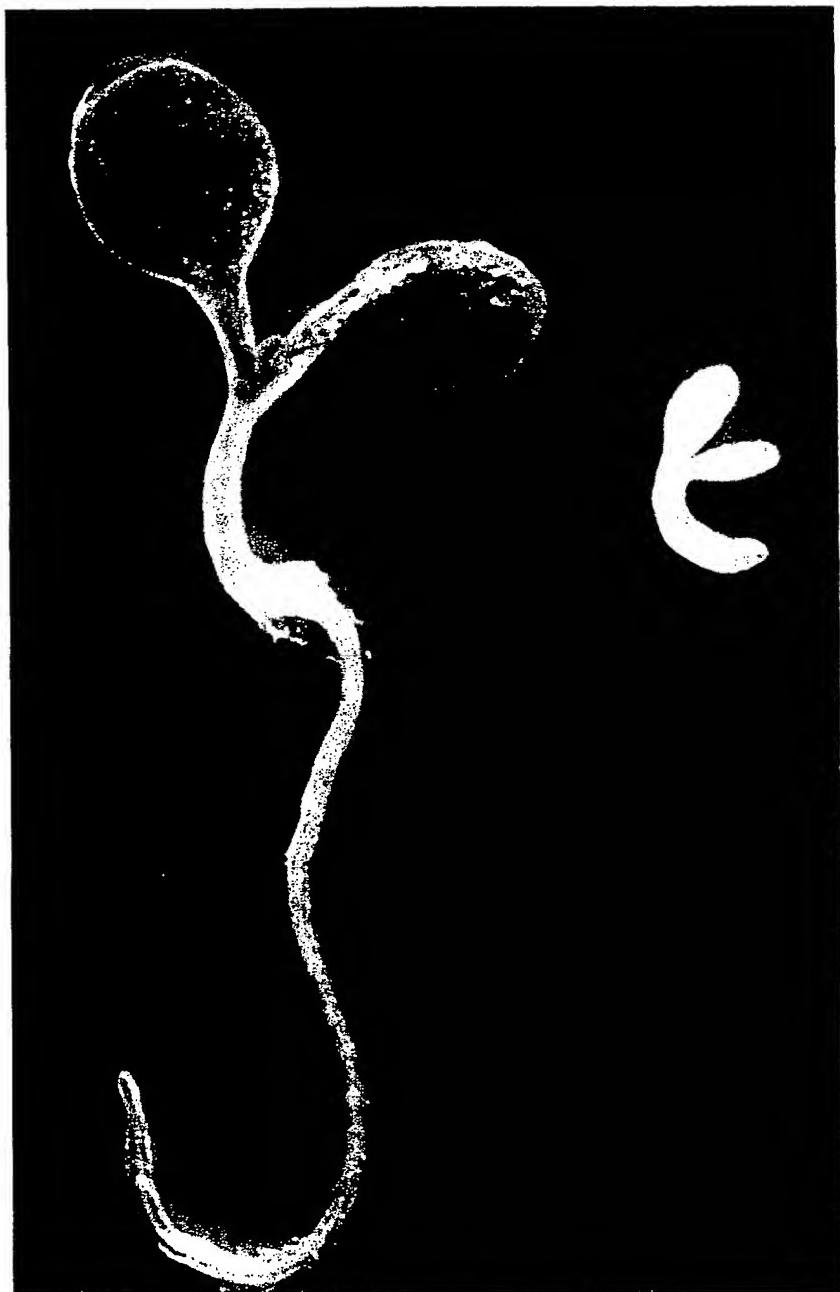


Fig. 1D

SUBSTITUTE SHEET (RULE 26)

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A+ HXK      KNOCK-OUT      SENSE A+ HXK

Fig. 2

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/04287

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :C07H 21/04; A01H 5/00; C12N 15/09, 15/14, 15/00  
 US CL : 435/455, 419; 536/23.1, 24.5; 800/419, 295

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/455, 419; 536/23.1, 24.5; 800/419, 295

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
 NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, MEDLINE, BIOTECHNO, SCISEARCH, BIOSIS, EMBASE, CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOUGHERTY et al. Transgenes and Gene Supression: Telling Us Something New? Current Opinion in Cell Biology. 1995. Vol. 7, pages 399-405, see entire document.	1-32
Y	HAMADA et al. Co-Suppresion of the Hdrophobin Gene HCf-1 is Correlated with Antisense RNA Biosynthesis in Cladosporium fulvum. Mol. Gen. Genet. 1998. Vol. 259, pages 630-638, see entire document.	1-32
Y,P	JENSEN et al. Cosuppression of I Transposon Activity in Drosophila by I-Containing Sense and Antisense Transgenes. Genetics. 1999. Vol. 153, pages 1767-1774, see entire document.	1-32

<input checked="" type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
*A*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	*T*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E*	earlier document published on or after the international filing date	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L*	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O*	document referring to an oral disclosure, use, exhibition or other means	*&*	document member of the same patent family
*P*	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search  20 APRIL 2000	Date of mailing of the international search report  21 JUN 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer  SEAN MCGARRY Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/04287

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CARTEA et al. Comparison of Sense and Antisense Methodologies for Modifying the Fatty Acid Composition of <i>Arabidopsis thalia</i> Oilseed. <i>Plant Science</i> . 1998. Vol. 136, pages 181-194, see entire document.	1-32